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New localities and lineages of the Atlas dwarf lizard *Atlantolacerta andreanskyi* identified using mitochondrial DNA markers

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Abstract

Atlantolacerta andreanskyi (Werner, 1929) is an endemic lizard from the High Atlas Mountains region of Morocco. A previous molecular assessment of this species using mitochondrial and nuclear DNA markers uncovered extensive genetic diversity with seven lineages indicative of a species complex. A morphological assessment of six of these lineages did not establish simple diagnostic features, and proposed these should be considered as a cryptic species, while highlighting the need for greater sampling across the range. In this study, we sampled 8 individuals from 5 previously unsampled localities and carried out genetic analyses to compare these populations to the known variation. Phylogenetic reconstruction based on mitochondrial DNA markers (12S rRNA and ND4) corroborates the previously described lineages and identified a new one. Interestingly, the two samples that account for this newly identified lineage have been collected from distinct localities – M'goun and Toumliline – that form a sister taxon to the population of Jbel Azourki.

Key Words

12S rRNA, Atlas Mountains, evolutionary history, Lacertidae, Morocco, ND4, phylogeny

Introduction

Atlantolacerta andreanskyi (Werner, 1929), is a lacertid lizard endemic to the High Atlas Mountains in Morocco, distributed across 440 km (straight line) from the western to the central areas of the massif (Bons and Geniez 1996; Mármol et al. 2019). It can be found in areas from 2400 m a.s.l. to 3800 m a.s.l., often under small rocks near watercourses and around the base of cushion-like thorny plants that offer a buffered microclimate (Werner 1929; Bons and Geniez 1996). The species presents a patchy spatial distribution, with populations separated by regions of unsuitable, lower elevation habitats.

Despite extensive herpetological surveys across the Atlas Mountains in recent years (e.g. Avella et al. 2019; Harris et al. 2010) there have been few additional populations reported, with minimal differences between the recent field guide and ones from much earlier (e.g. distribution reported

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from Mármol et al. 2019, compared to Bons and Geniez 1996). A molecular assessment of 8 populations widely distributed across the range using two mitochondrial and five nuclear markers (Barata et al. 2012) revealed extreme genetic diversity among seven of the eight populations analysed, demonstrating divergence levels indicating that *A. andreanskyi* should be considered a species complex.

A later assessment of phenotypic variability of this species, employing linear measurements, pholidotic and coloration characters in six of the previously analysed populations of *A. andreanskyi* (Barata et al. 2015), indicated that despite the high genetic divergence previously detected, morphological variation among populations was low. However, with almost every population studied representing a unique lineage, distribution of these cryptic forms remains essentially unknown.

The present study is the result of multiple additional fieldtrips to the region, and the collection of samples from additional localities to compare to the known genetic lineages. The aim was to increase the knowledge of the distribution of this species complex, and to include new samples within a phylogenetic framework.

Methods

All lizards were captured under permit from the High Commissariat for Water and Forests of Morocco. Fieldwork was carried out in the Spring of 2019 and 2022, and resulted in the identification of *A. andreanskyi* from 5 new localities (Fig. 1): 1) near the previously sampled population of Outabati (32°12'29.2"N, 5°27'25.2"W; circa 2660 m a.s.l.), 2) near Toumliline, a new locality over 30 km from any previously reported populations (31°54'46.1"N, 5°29'08.2"W; circa 2610 m a.s.l.), 3) near M'goun, the second highest peak of the Atlas Mountains (31°34'52.9"N, 6°16'11.8"W; circa 2710 m a.s.l.), south of the sampled populations of Jbel Azourki, 4) near Lake Tamda (31°18'59.55"N, 6°59'54.17"W), over 30 km East of the previously sampled Tizi n'Tichka population, and 5) north of the sampled populations in the furthest South, at Jbel Awlime (30°58'50.9"N, 8°45'18.7"W; circa 2540 m a.s.l.). Specimens were captured by hand and photographed for later comparison of external phenotypic differences between lineages. Tail tip samples were collected and preserved in tubes filled with ethanol 96%. All individuals were released at the place of capture.

Total genomic DNA was extracted from tail tip samples, using standard high-salt protocols (Sambrook et al. 1989). Eight new individuals were included, four from the population near Jbel Awlime, and one from each of the remaining new locations. Two mitochondrial DNA markers were amplified via polymerase chain reaction (PCR): 12S rRNA (12S) and partial NADH dehydrogenase 4 (ND4) and flanking tRNAs (tRNA-His), using previously published primers from Kocher et al. (1989) and Arévalo et al. (1994) respectively. The PCR thermocycler conditions used started with 95 °C for 10 min, followed by thirty-five cycles of 30 sec at 95 °C, 30 sec at 50 °C (12S) or 52 °C (ND4), and 30 sec at 72 °C with a final extension at 72 °C for 10 min. PCR success was assessed through electrophoresis, and single-band samples showing PCR product of the intended length were sent to GENEWIZ (Germany) for purification and standard Sanger sequencing. New sequences have been submitted to GenBank (Accession numbers OQ731434 to OQ731441 and OQ724509 to OQ724516).

The ND4 fragment was translated into amino acids in order to assess the reading frame and confirm the sequences corresponded to the expected protein. Available sequences from Barata et al. (2012) were included in the dataset, and sequences of *Podarcis tiliguerta* were included for outgroup purposes. All sequences were aligned using Muscle v5.0 (Edgar 2004). Estimates of phylogeny were produced by Bayesian Inference (BI) using MrBayes v3.2.6 (Ronquist and Huelsenbeck 2012), and Maximum Likelihood (MI) using MEGAX (Kumar et al. 2018). Best-fit nucleotide models and partitions schemes for the BI were selected using PartitionFinder2 (Lanfear et al. 2012) and were as follows: GTR+G for 12S, HKY+G+I, HKY+I, GTR+G respectively for each of the ND4 codon positions, and HKY+G+I for tRNAs. BI was run for 8×10^6 generations, with a sampling frequency of 1000 and all other parameters left as default. Best-fit nucleotide model for the concatenated ML analysis was selected using MEGAX built-in tool, selecting the HKY+G+I. Nodal support was assessed by bootstrapping with 8000 replicates.

Results

Both Bayesian Inference and Maximum Likelihood analysis produced almost identical topologies, mostly differing slightly in the shallow nodes within populations (Fig. 2). The exception is the Jebel Awlime clade, appearing as a sister clade to Outabati, Jbel Ayache, Jbel Azourki, M'goun, Tizi n'Tichka, and Lake Tamda for the BI (posterior probability = 0.83) as seen in Fig. 2, or as sister taxon to the Outabati and Jbel Azourki clade for the ML, albeit with less support (bootstrap value = 53). As expected, the major lineages identified by Barata et al. (2012) were again recovered. The individual from locality (1), near Outabati, was most closely related to individuals from Barata et al. (2012) from nearby, although divergence was notable (3.00 ± 0.62% (SE) with the ND4 marker, 0.33 ± 0.30% (SE) with the 12S rRNA marker). Likewise, the individual from Lake Tamda (4) was most closely related to the known population from Tizi n' Tichka, but again divergence was notable (5.80 ± 0.69% (SE) with the ND4 marker, 1.54 ± 0.69% (SE) with the 12S rRNA marker). Unexpectedly, the individuals from Toumliline (2) and M'goun (3), despite being geographically separated by more than 60 km formed a well-supported clade, distinct from the sister taxa population of Jbel Azourki (8.15 ± 0.95% (SE) with the ND4 marker, 3.34 ± 0.98% (SE) with the 12S rRNA marker). The four newly sequenced individuals from north of Jbel Awlime

Figure 1. A. Distribution map of *Atlantolacerta andreanskyi*. Coloured dots are based on the distribution map populations of Barata et al. (2012). White dots represent the distribution points of Bons and Geniez (1996). The yellow triangle represents a specimen sampled in the Tichka plateau (Avella et al. 2019). The localities with newly sampled individuals are identified with numbers (1–5): Outabati (1), Toumliline (2), M'goun (3), Lake Tamda (4) and Jbel Awlime (5); **B, C.** Typical habitat of *Atlantolacerta andreanskyi* in Jbel Awlime and in Toumliline, respectively.

(5) were strongly supported as a clade along with samples from Barata et al. (2012) from nearby, despite the equivocal placement of the lineage in the overall phylogeny. The overall estimate of relationships between lineages is concordant with Barata et al. (2012): the northern Outabati and Jbel Ayache populations are sister taxa with strong support; populations from Jbel Sirwa are sister taxa to those from Oukaimeden and Jbel Toubkal; and populations from the central region of Tizi n' Tichka and Jbel Azourki form a clade along with the newly identified lineage from M'goun and Toumliline.

Discussion

The earlier molecular study of Barata et al. (2012) clearly indicated that *A. andreanskyi* could be considered a potential species complex, with important conservation implications since six lineages were identified using both mitochondrial and nuclear markers. However, these authors highlighted that the mountainous habitat was difficult to sample, and that the potential for additional cryptic forms to exist was high. In this study, by including four additional localities, we have demonstrated that this is indeed the case,

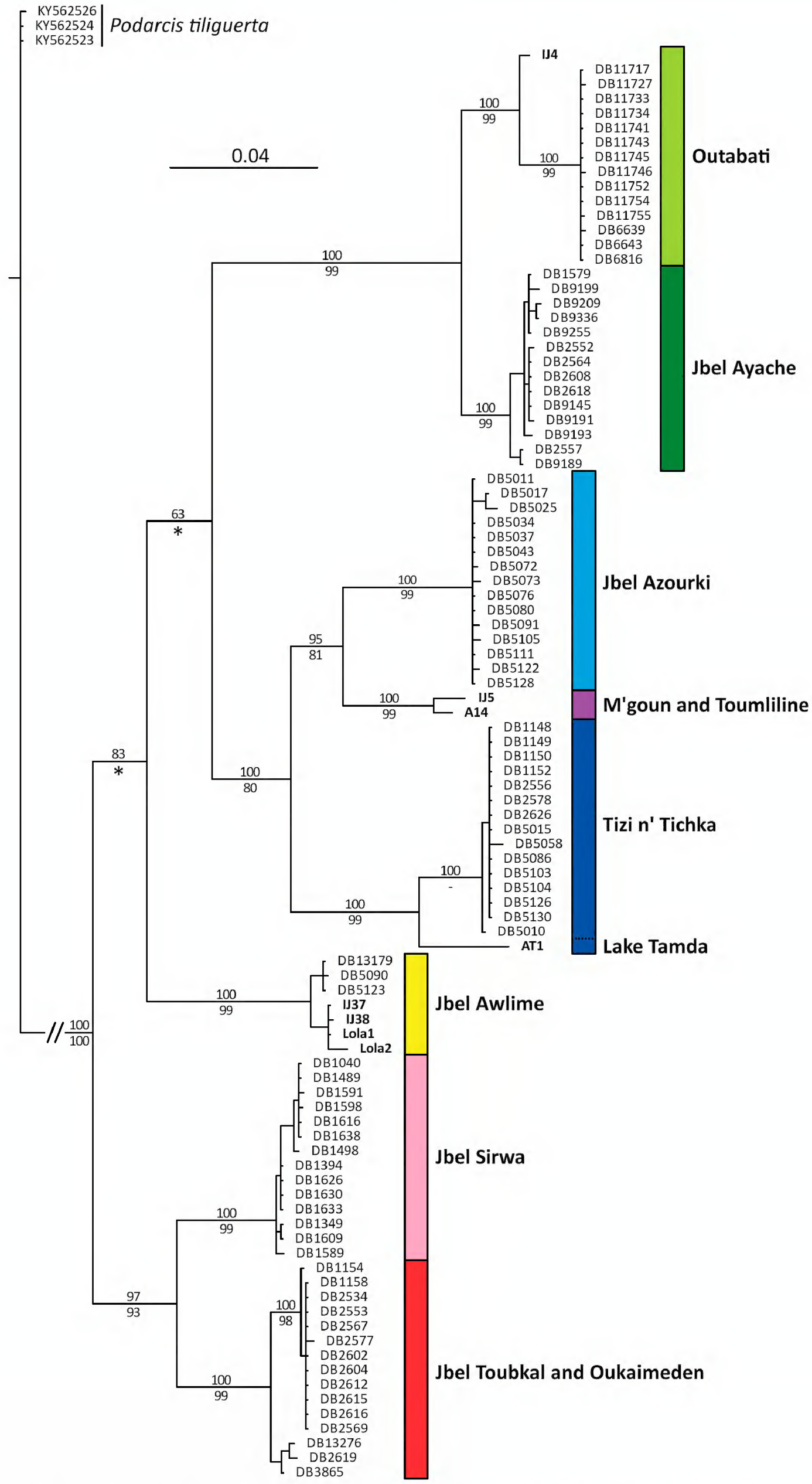

Figure 2. Bayesian tree estimate based on mitochondrial DNA sequences (12S and ND4 + tRNA-His) of *Atlantolacerta andreanskyi*. Bayesian posterior probabilities are shown above branches; Maximum Likelihood bootstrap values are shown below branches; asterisk indicate nodes not present on the ML tree estimate due to a different placement of the Jbel Awlime clade. The main lineages within the species are represented by distinct colours, corresponding to Figure 1. New samples used are named as IJ4 (1 – Outabati), IJ5 and A14 (2, 3 – Toumliline and M'goun), AT1 (4 – Lake Tamda), and IJ37, IJ38, Lola1 and Lola2 (5 – Jbel Awlime). Codes beginning "DB" are from Barata et al. (2012), in other cases GenBank numbers are indicated.

with a divergent lineage occurring in M'goun and Toumliline. The level of divergence, over 8% with the ND4 marker, is similar or higher than the variation between mountain species of the lacertid lizard genus *Iberolacerta* in the Pyrenees (Garcia-Porta et al. 2019), indicating another potential cryptic species within the *A. andreanskyi* complex.

Although Barata et al. (2015) reported some morphological differences between lineages, they also found that these could only be determined by examining multiple individuals and characters – simple diagnostic characters that could be used to classify specimens in the field were not identified. Colour pattern variation tended to match phylogenetic relationships, with the populations from Azourki and Tizi n'Tichka having a tendency towards more dark spots on the ventral head region, and a more intense ventral spotted pattern in general (Barata et al. 2015). The male specimen from Toumliline shows a similarly heavily spotted ventral pattern (Fig. 3), although with a single specimen observed, this clearly needs further assessment to see if this pattern variation is maintained within the new lineage.

In the far south of the range of *A. andreanskyi*, additional populations have recently been identified both in this study (locality 5) and in the Tichka plateau (Avella et al. 2019). Genetically, these seem to belong to the same lineage as those first identified from Jbel Awlime by Barata et al. (2012). This population has never been included in a morphological assessment, but based on the few specimens observed show a greatly reduced pigmentation pattern on the ventral (Fig. 3), similar to specimens in the occidental lineage (Oukaimeden and Jbel Sirwa) of Barata et al. (2015). The specimen collected from about 10 km northeast of the known Outabati population (locality 1), while clearly related to these, was also notably genetically distinct. The Outabati population shows a trend towards an absence of dorso-lateral lines in males (Barata et al. 2015), however, again with a single specimen observed it is not possible to assess if this feature is maintained in this new, nearby population. In much the same way, the specimen from Lake Tamda, while clearly related to the nearest assessed population from Tizi n'Tichka, was also genetically distinct using these mtDNA markers, highlighting just how much variation remains unknown.

In terms of comparative phylogeography, data is available for two other high mountain species of reptiles, the day gecko *Quedenfeldtia trachyblepharus*, and the atlas dwarf viper *Vipera monticola*. Both of these species, like *L. andreanskyi*, demonstrate divergent lineages in the Jbel Awlime region, with an endemic subspecies, *V. m. atlantica* recently described from there (Martínez-Freiría et al. 2021). While the divergences between *Q. trachyblepharus* and *L. andreanskyi* lineages are similar (both up to 9% for ND4 – this study and Harris et al. 2017), divergences between *V. monticola* lineages are much shallower. Furthermore, the number of identified lineages – 3 in *V. monticola*, 4 in

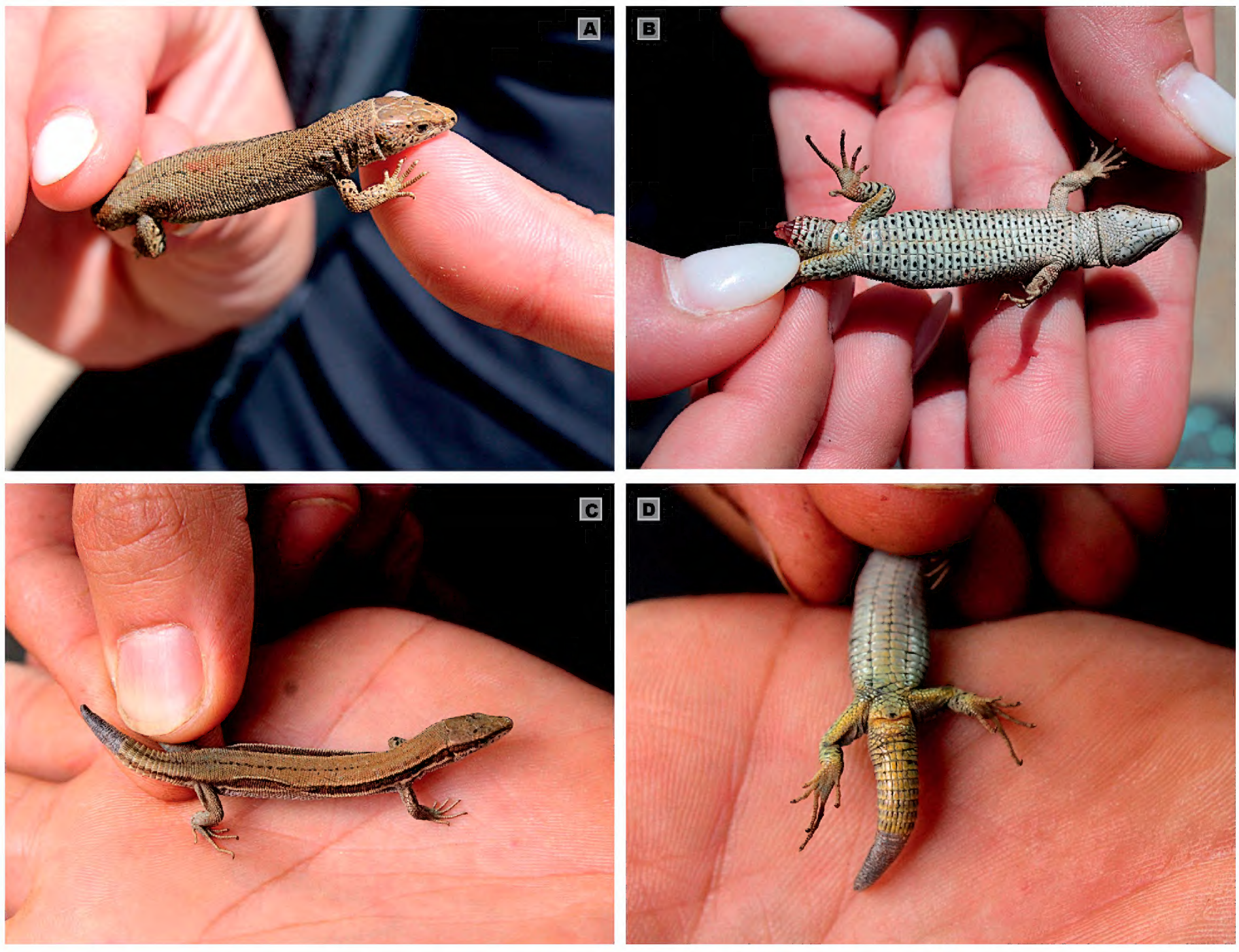

Figure 3. Male specimens of *Atlantolacerta andreanskyi* sampled in Toumliline (**A, B**) and Jbel Awlime (**C, D**).

Q. trachyblepharus and 8 in *L. andreanskyi* – also differs greatly, underlining the complexity not only within each species, but in elucidating general phylogeographic patterns.

Conclusion

Overall, our additional fieldwork within the range of *A. andreanskyi* confirms the expectation of Barata et al. (2012) that additional lineages occur, but also that single lineages can occur across larger areas. The identification of the population at Toumliline shows that the range of the *A. andreanskyi* species complex is greater than previously considered, and combined with the considerable diversity, and already known highlights, also shows how important it is to continue surveys across the region. Complete morphological assessments of unsampled lineages are needed to better determine morphological variation, even if this is slight, so that an integrated taxonomic revision can be performed. A recent assessment noted that the ecophysiological conservativeness of *A. andreanskyi* demonstrates its vulnerability to climate change (S'khifa et al. 2020, 2022), while also highlighting that low elevation populations are the most vulnerable. Detailed assessments of the range of lineages, including altitudinal ranges, will also therefore be essential to develop an appropriate conservation management plan for this cryptic species complex.

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